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Identification of Glucosylceramides Containing Sphingatrienine in Maize and Rice using Ion Trap Mass Spectrometry

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Running Head: Glucosylceramides containing sphingatrienine in maize and rice

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Abstract We characterized the glucosylceramide moieties from maize and rice using liquid chromatography-ion trap mass spectrometry. Glucosylceramides containing 4,8-sphingadienine (d18:2) acylated to hydroxy-fatty acids were detected as the predominant molecules both in maize and in rice. In addition, 4-hydroxy-8-sphingenine (t18:1) and sphingatrienine (d18:3) were found in maize and rice glucosylceramides, and in the case of rice, sphingenine (d18:1) was also detected. Glucosylceramides containing d18:3 were acylated to hydroxyl fatty acids (16 to 24 carbon atoms). Our results indicate the presence of the triene type of sphingoid base in higher plants.

Abbreviations

d18:0 Sphinganine
d18:1 Sphingenine
d18:2 Sphingadienine
d18:3 Sphingatrienine
t18:0 Phytosphingosine
t18:1 Hydoroxysphingeine
HPLC High-performance liquid chromatography
MS mass spectrometry

Introduction

Sphingolipids are found in a wide variety of organisms, and constitute a family of compounds that have a sphingoid base (long-chain base) with an amide-linked fatty acid and a polar head group. The hydrolyzed products of sphingolipids (ceramides and sphingoid bases) are highly

bioactive compounds that play roles as second messengers that are known to be involved in many aspects of cell regulation, such as cell growth, cell differentiation and apoptosis [1-3]. Recently, dietary sphingolipids have gained attention for their potential to protect the intestine from inflammation and cancer [4-9]. In addition, other physiological functions of sphingolipids, such as improving the barrier function of skin, lowering plasma lipids and prevention of melanin formation, have also been reported [10-12].

Diverse structures of the sphingoid base occur in nature. The most common sphingoid base of mammalian sphingolipids is sphingosine (*trans*-4-sphingenine, d18:1⁴). Smaller amounts of other sphingoid bases, such as sphinganine (dihydrosphingosine, d18:0) and phytosphingosine (4-hydroxysphinganine, t18:0) are frequently encountered. The structures of sphingoid bases in higher plants are more complicated than in mammals [13]. Plants primarily contain *cis*- and *trans*- isomers of Δ 8-unsaturated sphingoid bases, such as 8-sphingenine (d18:1⁸), 4,8-sphingadienine (d18:2^{4,8}) and 4-hydroxy-8-sphingenine (t18:1). Determination of those diverse structures including variations of the sphingoid backbone is important to understand the functional and nutritional significance of dietary sphingolipids.

Mass spectrometry is one of the most powerful methods for detecting and identifying the chemical structures of lipids including sphingolipids [14-16]. In this study, we characterized the structures of glucosylceramide, one of the predominant glycosphingolipids in plants, from rice and from maize using liquid chromatography-ion trap mass spectrometry. Our results demonstrate the presence of sphingatrienine (d18:3) in higher plants, which has been described previously in marine invertebrates [17-19] and was found in tobacco leaf [20].

Experimental Procedures

1 Materials

2 Glucosylceramides were prepared from maize grain and from rice grain using a silica gel column
3 after lipid extraction and saponification as described previously [21]. All other chemicals and
4 solvents were of reagent grade.

5

6 LC-MS/MS analyses

7 An HPLC system coupled to LCMS-IT-TOF equipped with an electrospray ionization interface
8 (Shimadzu, Kyoto, Japan) was used. A TSK gel ODS-100Z column (2.0 x 50 mm, 3 μ m, Tosoh,
9 Tokyo, Japan) was eluted with acetonitrile/water (93:7, v/v) at a flow rate of 0.2 mL/min. The
10 MS was operated with the following conditions: probe voltage of 4.50 kV, CDL temperature of
11 200°C, block heater temperature of 200°C, nebulizer gas flow of 1.5 L/min, ion accumulation
12 time of 100 msec, MS range of m/z 650 to 900, MS² range of m/z 200 to 300, and CID
13 parameters as follows: energy, 60%; collision gas 60%. For the structural analysis of
14 glucosylceramide, [M+H-18]⁺ (loss of water) in the positive scan mode was used for MS/MS
15 analysis to obtain the product ions. The typical signals which are characteristic for the
16 sphingoid base moieties were observed as the product ions using the auto MS/MS detection
17 mode. In this system, product ions corresponding to d18:1, d18:2 and d18:3 were m/z 264.3,
18 m/z 262.3 and m/z 260.2, respectively [14-16]. In the case of glucosylceramide molecules
19 consisting of t18:1, the loss of glucose [M+H-162]⁺ was used as the precursor ion and the
20 product ions corresponding to t18:1 were m/z 280.3 and m/z 262.3 [16]. Pairs of the
21 structurally specific product ions of sphingoid bases and their precursor ions were used for
22 the identification of glucosylceramide molecules.

23 Results and Discussion

1 In the positive full scan mode, $[M+Na]^+$, $[M+H]^+$ and $[M+H-H_2O]^+$ were the predominant
2 signals in each peak. It is well known that the sugar moiety of glycosylceramides in plants is
3 mostly glucose [22]. In the case of molecules consisting of t18:1, the loss of glucose
4 $[M+H-162]^+$ was also clearly detected. Glucosylceramide molecules containing d18:2 and
5 t18:1 were determined both in maize and in rice as described previously (Fig. 1A and 2A) [16].
6 In the case of rice glucosylceramide, molecules consisting of d18:1 were also identified.
7 Detection of glucosylceramide consisting of d18:2 and t18:1 was separated into two peaks, *cis*-
8 and *trans*- isomers of $\Delta 8$ -unsaturated sphingoid bases. Cis-isomer was detected earlier than
9 *trans*-isomer by separation of reverse phase [20]. Predominantly hydroxy fatty acids containing
10 16 to 26 carbon atoms were detected both in maize and in rice glucosylceramides.

11 We verified that the characteristic product ion at m/z 260.2 corresponding to d18:3 was
12 detected in maize glucosylceramide using the auto MS/MS detection mode (Fig. 1A). Five
13 peaks in the total ion chromatogram of maize glucosylceramide showed the product ion at m/z
14 260.2 (peak 1-5 in Fig. 1A). The MS spectra of those 5 peaks are shown in Figure 1B-F. As
15 the precursor ion of m/z 260.2, $[M+H-18]^+$ ions at m/z 694.5, 722.5, 750.6, 778.6 and 806.6 were
16 detected. The identification of each peak component is summarized in Table 1. The acylated
17 fatty acid moieties were hydroxy fatty acids with 16-24 carbon atoms. In the case of rice,
18 glucosylceramide consisted of d18:3-C18:0h and d18:3-C20:0h (Fig. 2 and Table 1).

19 It has been reported that the sphingoid bases in marine invertebrates are quite different
20 from those in mammals and in plants [13]. Triene bases with conjugated diene, such as
21 2-amino-4,8,10-octatriene-1,3-diol (d18:3) and 2-amino-9-methyl-4,8,10-octatriene-1,3-diol
22 (d19:3), were identified in marine invertebrates including ascidians [17], starfish [18, 19] and
23 squid [23] and also some fungi [24]. We have also reported that sea cucumber

glucosylceramide has sphingoid bases with three double bonds [25]. . Sperling et al. described the presence of sphingatrienine in tobacco leaf by HPLC analysis of sphingoid base derivatized with dinitrophenyl [20]. In this study, we identified several molecular species of sphingatrienine-containing glucosylceramides in maize and rice by LC-MS/MS system. However, the locations of double bonds in sphingatrienine structure have not been identified. Sphingolipids of plant organisms contain primarily d18:1⁸, d18:2^{4,8} and t18:1⁸ as sphingoid bases and sphingolipid Δ 4-desaturase and sphingolipid Δ 8-desaturase have been identified in plants [22]. It has been reported that the composition of sphingoid bases differs between chilling sensitive and tolerant plants [26]. Details of tissue distribution, synthetic pathways and functions of plant sphingatrienines remain to be elucidated.

Recently, dietary sphingolipids have gained attention for their potential to protect the intestine from inflammation and cancer [4-9]. We reported that the daily intake of plant-origin glucosylceramides in Japan is estimated to be 50 mg due to their presence in foodstuffs [27] and we investigated the digestion and absorption of plant-derived sphingolipids [21]. Our findings indicate that the metabolic fate of plant-derived sphingoid bases, such as 4,8-sphingadienine, within enterocytes differs from that of sphingosine. Sphingoid bases, except for sphingosine, appear to be transported out of cells across the apical membranes of enterocytes by P-glycoprotein after absorption and consequently the intestinal uptake is quite poor [28, 29]. Thus, the determination of sphingolipid structures, including variation of the sphingoid backbone, from dietary sources is important to understand their functional and nutritional significance.

In this study, we analyzed the chemical structures of glucosylceramides from maize and from rice using liquid chromatography-ion trap mass spectrometry. Our results indicate the presence of sphingatrienine (d18:3) in higher plant sphingolipids. MS/MS analysis is a

1 powerful method to identify the molecular structures of sphingolipids from biological sources.

2

3

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6

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Table 1 Glucosylceramides containing sphingatrienine (d18:3) from maize and from rice identified by HPLC-MS/MS analysis.

Peak No. in Fig. 1 and 2	Precursor ion [M+H] ⁺	m/z [M+H-18] ⁺	Product ion m/z	Molecule	Source
1	712.5	694.5	260.2	d18:3-C16:0h	Maize
2	740.5	724.5	260.2	d18:3-C18:0h	Maize, Rice
3	768.6	750.6	260.2	d18:3-C20:0h	Maize, Rice
4	796.6	778.6	260.2	d18:3-C22:0h	Maize
5	824.6	806.6	260.2	d18:3-C24:0h	Maize

1 **Figure Legends**

2

3 FIG. 1. Total ion and selected ion chromatograms of maize glucosylceramide (A) and mass
4 spectra of peak components (B-F).

5

6 FIG. 2. Total ion and selected ion chromatograms of rice glucosylceramide (A) and mass
7 spectra of peak components (B and C).



